Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses

Ana-Maria Bulau[a], Marcel F. Nold[b], Suzhao Li[c], Claudia A. Nold-Petry[b], Michaela Fink[a], Ashley Mansell[d], Tobias Schwerd[a], Jaewoo Hong[e], Anna Rubartelli[f], Charles A. Dinarello[c,g,1], and Philip Bufler[a,1]

[a]Department of Pediatrics, Dr. von Hauner Children's Hospital, Ludwig Maximilians University, D-80337 Munich, Germany; [b]Ritchie Centre and [d]Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Monash University, Melbourne, VIC 3168, Australia; [c]University of Colorado Denver, Aurora, CO 80045; [e]Department of Biomedical Science and Technology, Konkuk University, Seoul 143-701, Korea; [f]Cell Biology Unit, Istituto Di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliera Universitaria San Martino, Istituto Scientifica (per lo studio e la cura dei) Tumori, 16132 Genoa, Italy; and [g]Department of Medicine, Radboud University Medical Centre, 6500 HB, Nijmegen, The Netherlands

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IL-37 is a fundamental inhibitor of innate immunity. Human IL-37 has a caspase-1 cleavage site and translocates to the nucleus upon LPS stimulation. Here, we investigated whether caspase-1 processing affects IL-37–mediated suppression of LPS-induced cytokines and the release from cells by analyzing a caspase-1 cleavage site mutant IL-37 (IL-37D20A). Nuclear translocation of IL-37D20A is significantly impaired compared with WT IL-37 in transfected cells. LPS-induced IL-6 was decreased in cells expressing WT IL-37 but not IL-37D20A. The function of IL-37 in transfected bone marrow-derived macrophages is nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome-dependent, because IL-37 transfection in apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain- and NLRP3-deficient cells does not reduce levels of IL-6 and IL-1β upon LPS stimulation. IL-37–expressing macrophages release both precursor and mature IL-37, but only the externalization of mature IL-37 was dependent on ATP. Precursor and mature IL-37 was also secreted from human dendritic cells and peripheral blood mononuclear cells. To determine whether IL-37 is active in the extracellular compartment, we pretreated IL-37 transgenic mice with IL-37–neutralizing antibodies before LPS challenge. In IL-37–expressing mice, neutralizing IL-37 antibodies reversed the suppression of LPS-induced serum IL-6. In contrast, the addition of neutralizing antibody did not reverse suppression of LPS-induced IL-6 in mouse macrophages transfected with IL-37. Although caspase-1 is required for nuclear translocation of intracellular IL-37 and for secretion of mature IL-37, the release of the IL-37 precursor is independent of caspase-1 activation. IL-37 now emerges as a dual-function cytokine with intra- and extracellular properties for suppressing innate inflammation.

With the exception of the IL-1 receptor antagonist, members of the IL-1 family are first synthesized as precursor molecules containing a propeptide domain lacking a classical signal sequence (1). Caspase-1 has emerged as the main intracellular processing enzyme responsible for maturation of active IL-1β and IL-18, which are then released into the extracellular space, as shown for IL-1β and IL-18 (2, 3). The IL-1 family member IL-37 is also synthesized as a precursor and is processed to its mature form upon LPS treatment (4, 5). Caspase-1 seems to be the main enzyme responsible for the in vitro maturation of IL-37 in comparison to caspase-4 and granzyme B (4). A putative cleavage site for caspase-1 is located in exon 1 between residues D20 and E21 of IL-37 (4). HEK 293 or CHO cells transfected with the IL-37 precursor release IL-37 starting at amino acid V46, suggesting a second cleavage site in the sequence encoded by exon 2 (6). We previously demonstrated that processing of IL-37 is only partially inhibited by caspase-1 inhibitors, indicating that caspase-1 is not the only enzyme responsible for the processing of IL-37 (5).

In our previous study, we showed that transgenic expression of human IL-37 in a mouse macrophage line significantly suppressed the production of proinflammatory cytokines and chemokines (5). Furthermore, we reported that IL-37 has significant anti-inflammatory effects in an in vivo model of septic shock and dextran sulfate sodium salt-induced colitis (7, 8). Here, we investigate the role of caspase-1 processing on the cytokine-suppressing function of IL-37. We introduced a point mutation into the caspase-1 cleavage site in the IL-37 gene by site-directed mutagenesis and expressed mutant IL-37 in RAW264.7 (RAW) mouse macrophages. In addition, we studied the release of IL-37 from human peripheral blood mononuclear cells (PBMCs) and dendritic cells (DCs). The data indicate that the precursor and mature forms of IL-37 are secreted from activated cells upon inflammasome activation and that caspase-1 processing of IL-37 is important for its anti-inflammatory activity in vitro and in vivo.

Results

Mutation of Caspase-1 Cleavage Site Reduces Processing of IL-37. We predicted a caspase-1 cleavage site in IL-37 between residues D20 and E21 of exon 1 and generated a mutant form of IL-37 by site-directed mutagenesis of aspartic acid at position 20 (D) to alanine (IL-37D20A). RAW cells were transfected with the mutant IL-37 inserted into pTarget expression plasmid. Respective single-cell clones were generated expressing either WT IL-37 or IL-37D20A and stimulated with LPS. As shown in Fig. 1*A*, cells

Significance

IL-37 exerts broad inhibitory properties on the innate inflammatory and acquired immune responses. We mutated the caspase-1 site in IL-37 and show that caspase-1 processing is required for maturation of the intracellular IL-37 precursor for its translocation to the nucleus. Because nuclear translocation of IL-37 is required for the suppression of LPS-induced IL-6, the data define a unique consequence for caspase-1 inhibition, that is, reversal of the anti-inflammatory activities of endogenous IL-37. In addition, neutralizing antibodies reverse the suppression of LPS-induced IL-6 in IL-37 transgenic mice, supporting a role for extracellular signaling by IL-37. Thus, similar to IL-1α and IL-33, IL-37 now emerges as a dual-function cytokine with both intra- and extracellular mechanisms of action.

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The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. E-mail: cdinarello@mac.com or philip.bufler@med.uni-muenchen.de.

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expressing the WT IL-37 contain mostly the precursor form, but upon LPS stimulation, the mature form is detectable; however, the mature form is markedly reduced in cells expressing the IL-37D20A mutant. We also expressed WT IL-37 and IL-37D20A in the p-enhanced CFP-C1 vector with CFP attached to the N terminus of IL-37, allowing a better size separation of pro- and mature IL-37 on SDS/PAGE. We depicted similar data with decreased expression of mature IL-37 following LPS stimulation in the cells expressing IL-37D20A (Fig. 1*B*).

Nuclear Translocation and Anti-Inflammatory Function of IL-37 Compared with IL-37D20A. Because nuclear translocation of IL-37 is inhibited by caspase-1 inhibition (5), we investigated whether nuclear translocation is reduced in IL-37D20A–transfected cells. We used two different methods, cell fractionation and confocal microscopy. Fig. 2*A* shows that only nuclear extracts from RAW cells expressing WT IL-37 contain IL-37, whereas upon stimulation with LPS, there is increased synthesis of IL-37 but nuclear extracts from cells expressing IL-37D20A reveal almost no detectable IL-37. We next examined nuclear translocation using confocal microscopy and IL-37 that contained YFP at the carboxyl terminus by the use of p-enhanced YFP (pEYFP)-N1 expression plasmid (IL-37–YFP). As shown in Fig. 2*B*, whereas WT IL-37 translocates to the nucleus of transfected RAW cells upon LPS treatment (Fig. 2*B*, *Left*), mutated IL-37D20A is restricted to the cytoplasm (Fig. 2*B*, *Right*).

Next, we examined whether the suppression of LPS-induced IL-6 by cells expressing IL-37 is impaired by the mutation of the caspase-1 site. As expected, levels of IL-6 were reduced by 70% in cells expressing WT IL-37 compared with LPS-stimulated mock-transfected cells. In contrast, there was no suppression of IL-6 in cells expressing the mutant IL-37D20A (Fig. 2*C*).

Deletion of Nucleotide-Binding Oligomerization Domain-Like Receptor Family, Pyrin Domain-Containing 3 or Apoptosis-Associated Speck-Like Protein Containing a Carboxy-Terminal Caspase Recruitment Domain Abrogates the Anti-Inflammatory Property of IL-37. Caspase-1 can be activated via the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome. To study the caspase-1–dependent functionality of IL-37 in greater detail, we investigated whether the integrity of the NLRP3 inflammasome is required for the suppression of LPS-induced IL-6 and IL-1β in cells transfected with IL-37. We transfected immortalized bone marrow-derived macrophages from WT mice, apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC)-deficient mice, and NLRP3-deficient mice [generated as described by Hornung (9)] with an IL-37 expression or empty control plasmid and stimulated the cells with LPS or vehicle. Costimulation of cells by the transfection procedure followed by LPS administration was sufficient to activate the inflammasome. As expected, WT cells expressing nonmutated WT IL-37 released significantly reduced levels of IL-6 and IL-1β compared with cells transfected with the control vector (Fig. 3*A*). In ASC- and NLRP3-deficient macrophages, there was no suppression of LPS-induced IL-6 and IL-1β by IL-37 (Fig. 3 *B* and *C*). There was no detectable IL-1β in the vehicle-treated cells.

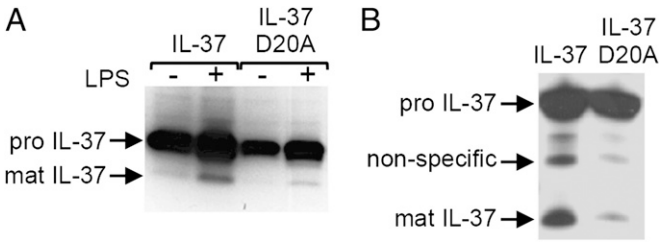

Fig. 1. Mutation of caspase-1 cleavage site reduces processing of IL-37 after LPS stimulation. Single-cell clones of stable RAW transfectants expressing either IL-37 or IL-37D20A alone (*A*) or as a fusion protein with an N-terminal CFP tag (*B*) were challenged for 18 h with LPS (100 ng/mL). Cell lysates (total of 50 μg of protein per lane) were subjected to Western blotting using a murine mAb against human IL-37. A representative Western blot of four experiments is shown. mat, mature.

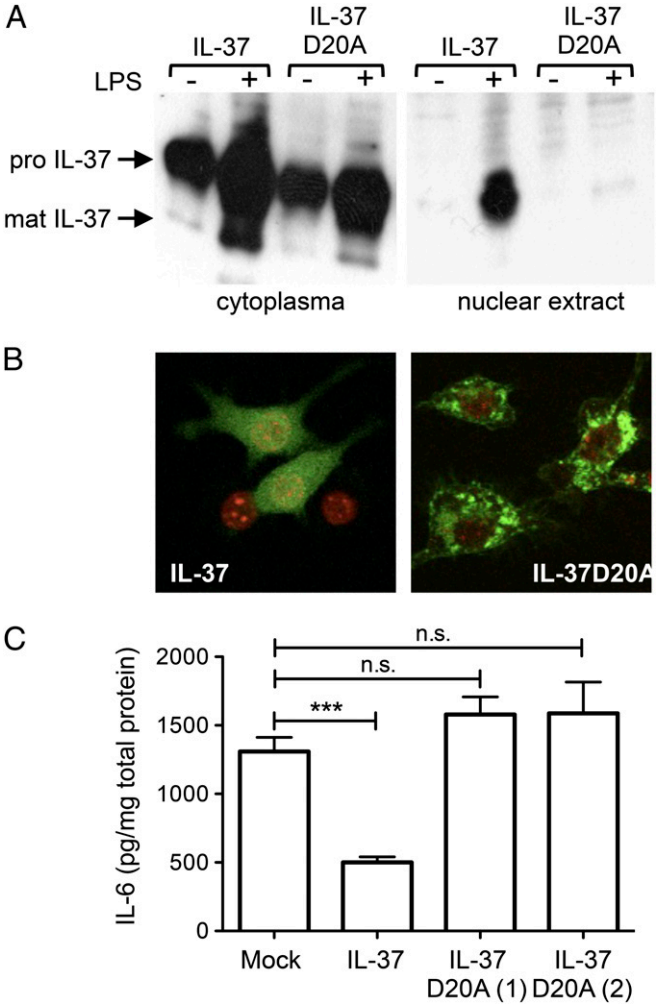

Fig. 2. Nuclear translocation and anti-inflammatory function of IL-37 compared with IL-37D20A. (*A*) Cytoplasmic and nuclear extracts from RAW cells expressing IL-37 and IL-37D20A without or with 18 h of LPS stimulation (100 ng/mL) were subjected to Western blotting with monoclonal mouse anti-human IL-37. IL-37 in the nuclear extract is the mature form, which is running at a slightly higher molecular weight on the gel due to nucleic acid contamination in the nuclear protein sample. (*B*) Subcellular distribution of IL-37 and IL-37D20A as a fusion protein with a carboxyl-terminal tag 18 h after LPS stimulation (100 ng/mL) in transfected RAW cells. Red indicates nuclear staining, and green indicates IL-37 staining. One of three representative experiments performed is shown (Magnification: 100×). (*C*) Two single-cell clones of stable RAW transfectants expressing IL-37D20A (1, 2), one clone expressing WT IL-37, and one mock-transfected clone were stimulated with LPS (100 ng/mL) for 18 h. Mean ± SEM. IL-6 levels in supernatants of stimulated cells of four independent experiments are shown. ***$P < 0.001$ for mock-transfected vs. IL-37–transfected cells. n.s., not significant.

Release of IL-37 from RAW Macrophages, PBMCs, and DCs. The mechanism of IL-37 externalization remains unresolved. ATP-dependent release and maturation upon activation of the NLRP3 inflammasome and caspase-1 cleavage are described well for the precursor forms of IL-1β and IL-18 (10, 11). We

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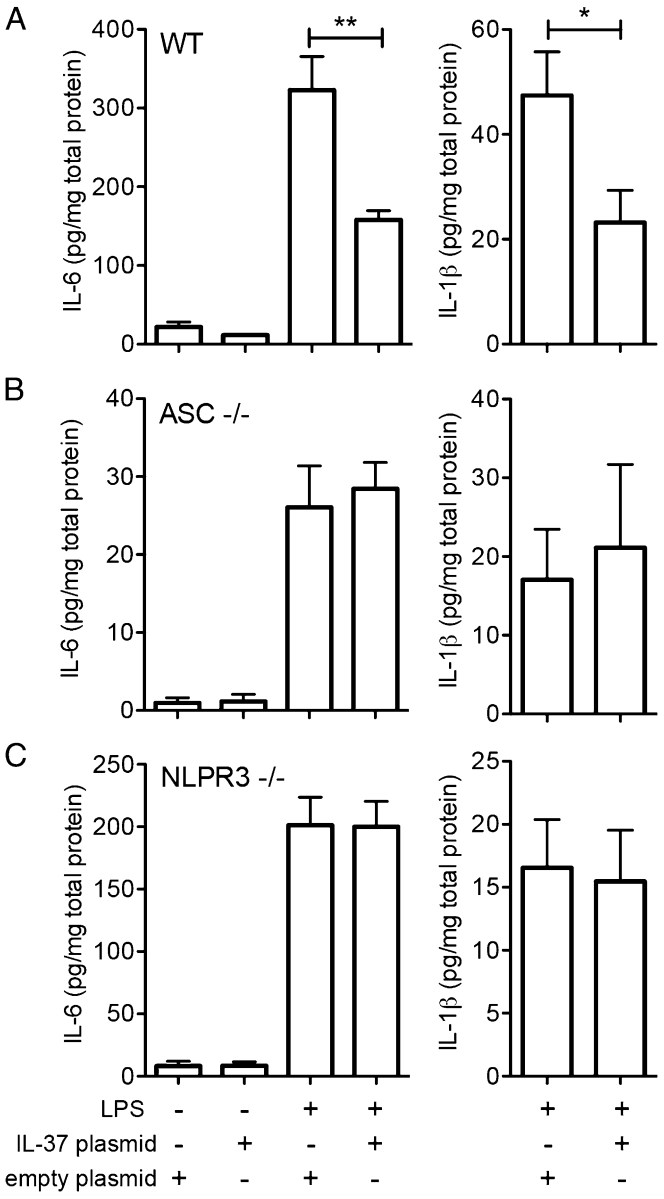

Fig. 3. NLRP3 and ASC are required for the anti-inflammatory function of IL-37. Immortalized bone marrow-derived macrophages from WT mice (*A*) or ASC-deficient mice (ASC$^{-/-}$) (*B*) and NLRP3-deficient mice (NLPR3$^{-/-}$) (*C*) (9) were transfected with an IL-37 expression vector or an empty vector for control and then stimulated with LPS (100 ng/mL) or vehicle for 24 h. Mean ± SEM abundance of IL-6 and IL-1β (ratio of cytokines per milligram of protein of cell lysates) in the supernatants is shown ($n = 5$). *$P < 0.05$ and **$P < 0.01$ for empty vector vs. IL-37 expression plasmid.

therefore investigated whether this mechanism also accounts for the release of IL-37. As depicted in Fig. 4*A*, precursor IL-37 is released into the supernatant of transfected RAW cells after LPS stimulation and 5–10 min of exposure to ATP. Release of mature IL-37 occurred only upon prolonged exposure to ATP for 15–20 min and was markedly reduced by a pan-caspase inhibitor. Similar to transfected RAW cells, human PBMCs also release IL-37 upon stimulation with LPS and ATP (Fig. 4*B*). In addition, Fig. 4*C* shows the presence of IL-37 in lysates of human DCs even in the absence of exogenous stimulation. However, upon stimulation with agonist antibodies against CD94, HLA-I, and intercellular adhesion molecule 1, there was induced secretion of both pro- and mature IL-37.

Natural Killer Cell/DC Interaction Induces IL-37 Clustering to the Immunological Synapse. IL-37 might be released directly into the immunological synapse, in which case the cytokine acts in an autocrine or paracrine manner via receptors in close proximity to the source of the cytokine. We therefore analyzed the subcellular distribution of IL-37 in DCs in an established coculture with autologous natural killer (NK) cells (12). Fig. 5 (*Upper* and *Lower*) displays intense cytoplasmic staining for IL-37 of a DC interacting with several NK cells in two focus layers. IL-37 staining in the DC polarizes in the direction of the NK cells at the focus layer corresponding to the immunological synapse.

Antibodies to Human IL-37 Reverse the Suppression of LPS-Induced IL-6 in Vitro and During Endotoxemia in Vivo. Because both mature IL-37 and the IL-37 precursor are released from activated cells, we asked whether IL-37 in the extracellular space suppresses cytokine production. First, we determined whether monoclonal anti–IL-37 neutralized recombinant IL-37 and prevented the reduction in IL-6 from LPS-stimulated human M1 macrophages in vitro. As shown in Fig. 6*A*, M1 macrophages pretreated with recombinant IL-37 and then stimulated with LPS released less IL-6 compared with M1 cells pretreated with vehicle (zero line). This reduction was not observed when recombinant IL-37 was first mixed with the monoclonal anti–IL-37 and then

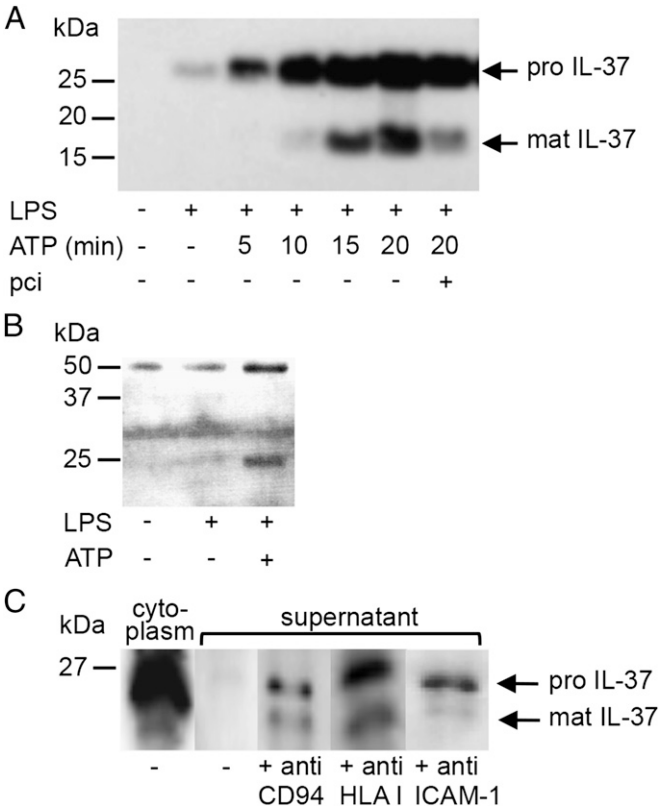

Fig. 4. Release of IL-37 from RAW macrophages, PBMCs, and DCs. (*A*) RAW–IL-37 cells were stimulated with LPS (100 ng/mL) or vehicle for 4 h and then subjected to a 20-min pulse of ATP (5 mM), as indicated, in the presence or absence of a pan-caspase inhibitor (pci, 10 μg/mL), followed by immunoblotting for IL-37. (*B*) PBMCs (5×10^6 cells) were stimulated with LPS (100 ng/mL) or vehicle for 4 h, followed by treatment with ATP (5 mM) for 15 min. Supernatants of RAW–IL-37 cells and PBMCs were harvested, concentrated, and analyzed by Western blotting for the presence of IL-37. (*C*) Western blot analysis with murine anti-human IL-37 antibody of cytoplasmic extracts and concentrated supernatants of DCs stimulated with vehicle or anti-CD94, anti-HLA I, or anti-intercellular adhesion molecule 1 (ICAM-1) antibodies. One of three to four representative experiments performed is shown.

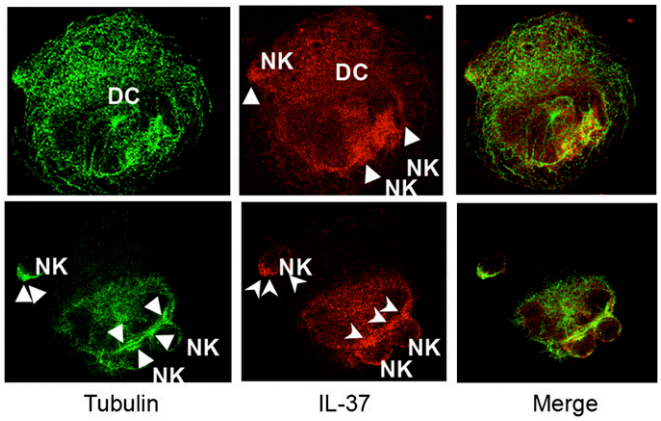

Fig. 5. NK cell/DC interaction induces IL-37 clustering to the immunological synapse. Confocal microscopy analysis of tubulin (green) and IL-37 (red) in DC/NK cell cocultures after 6 h of incubation. (*Upper* and *Lower*) Two different focus layers of the same DC/NK cell conjugate are shown. Arrowheads and triangles indicate the immunological synapse where IL-37 and tubulin polarize together. One of four representative experiments performed is shown. (Magnification: 100×.)

added to the M1 macrophages. In fact, neutralization of the recombinant IL-37 actually resulted in significantly more IL-6 after LPS stimulation compared with IL-37 mixed with a control isotype antibody. This increase suggests that the anti–IL-37 neutralizes the recombinant IL-37 but may also neutralize the endogenous IL-37 produced by M1 macrophages. Neutralization of endogenous IL-37 is consistent with the report that IL-37 depletion in human PBMCs results in increased LPS and IL-1β–induced cytokines (8).

To test the hypothesis that IL-37 released in IL-37 transgenic (IL-37tg) mice during endotoxemia is active in suppressing cytokine production, we pretreated IL-37tg mice with the monoclonal anti–IL-37 before administration of LPS. As shown in Fig. 6*B*, plasma levels of IL-6 increased compared with the effect of a control mouse isotype antibody. We repeated this experiment by pretreating IL-37tg mice with an affinity-purified goat antibody against human IL-37 before LPS. Pretreatment with anti–IL-37 resulted in a small increase in the level of circulating IL-6 compared with a control IgG in WT C57BL/6 mice (Fig. 6*C*). In contrast, there was a threefold increase in serum IL-6 after LPS in IL-37tg mice that received anti–IL-37 compared with control IgG (Fig. 6*C*).

Overexpression of IL-37 in either human macrophages or mouse RAW cells reduces in vitro cytokine production in response to LPS (5, 8). We therefore asked whether anti–IL-37 prevented this reduction in IL-37–transfected RAW cells. As shown in Fig. S1, the affinity-purified goat anti–IL-37 did not affect the suppression of LPS-induced IL-6 in IL-37–expressing RAW cells. These data indicate that the primary anti-inflammatory effect of IL-37 in transfected RAW macrophages is via nuclear translocation and is independent of extracellular IL-37.

Discussion

IL-37 reduces disease severity in a broad spectrum of innate responses by preventing excessive inflammation. To date, the data supporting the anti-inflammatory properties of IL-37 are derived from cells transfected with the precursor form of the human gene, a knockdown of endogenous IL-37 in primary human cells, and from studies using a strain of mice expressing human precursor IL-37 or mice injected with recombinant human IL-37 (1, 5, 8, 13). Similar to IL-18 and IL-1β, the precursor form of IL-37 contains a predicted caspase-1 cleavage site for processing into a mature cytokine (4). However, the functional relevance of processing by the caspase-1 site in the IL-37 precursor remains unknown. Therefore, we generated a mutant form of IL-37 by introducing a point mutation at the cleavage site for caspase-1, which is located on exon 1 between residues D20 and E21. This caspase-1 cleavage site generates an N terminus exactly nine amino acids upstream from the IL-1 family consensus sequence (A-X-D), as shown for IL-1β and IL-18 (14, 15).

As expected, RAW cells transfected with mutated IL-37D20A express less mature IL-37 compared with cells expressing WT IL-37. However, there was no complete inhibition of IL-37 maturation in IL-37D20A–expressing cells, indicating that alternative caspase-1 cleavage sites or additional proteases may be involved in the processing of IL-37 (4). We previously reported that a caspase-1

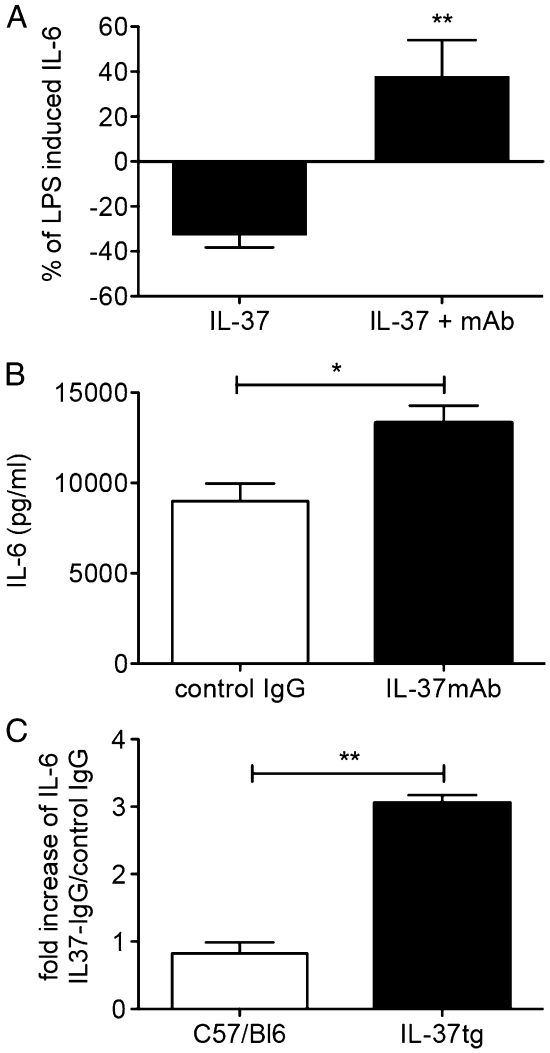

Fig. 6. Anti–IL-37 abrogates the protective effect of IL-37 in endotoxemia. (*A*) Human M1 macrophages were incubated with recombinant precursor IL-37 (1 ng/mL) premixed with 50 ng/mL mAb or the isotype control IgG for 1 h at room temperature and then added to the cells. After 2 h, LPS (10 ng/mL) was added, and supernatant IL-6 was determined after 24 h of incubation. Mean ± SEM percentage of change in IL-6 abundance is depicted. $^{**}P < 0.01$ for IL-37 vs. IL-37 plus blocking antibody. (*B*) IL-37 transgenic mice were injected with the mAb against IL-37 or control IgG2B (100 μg) 3 h before LPS injection (100 μg per mouse, $n = 5$ mice per group). Mean ± SEM serum levels of IL-6 after 4 h of LPS treatment are shown. $^{*}P < 0.05$ for IL-37 mAb vs. control IgG. (*C*) IL-37 transgenic and WT C57/Bl6 mice were injected with preimmune goat IgG (control) or polyclonal anti–IL-37 (200 μg) 3 h before LPS injection (100 μg per mouse, $n = 3$ mice per group). IL-6 was measured in sera after 4 h of LPS treatment. Data are expressed as fold increases of IL-6 (mean ± SEM) in mice treated with anti–IL-37 vs. control IgG. $^{**}P < 0.01$ for C57/Bl6 vs. IL-37tg mice.

inhibitor reduced the nuclear translocation of an IL-37–carboxyl terminal YFP fusion protein into transfected RAW cells (5). Here, we demonstrate that nuclear translocation is also reduced in RAW cells transfected with IL-37D20A. This observation underlines the essential role of a caspase-1–dependent cleavage step for nuclear entry of IL-37.

We next investigated whether mutant IL-37D20A retains its ability to reduce LPS-induced IL-6, as does WT IL-37 (5, 8). Indeed, levels of LPS-induced IL-6 were significantly increased in RAW–IL-37D20A cells compared with WT IL-37–expressing cells. These data support the concept that caspase-1 processing at position D20 activates IL-37 for its anti-inflammatory function in transfected cells. Caspase-1 is the effector molecule of the NLRP3 inflammasome required for processing the inactive precursors of IL-1β and IL-18 into biologically active cytokines (16). The net effect of caspase-1 during the response to infection depends on the presence of the IL-1β and IL-18 precursors as the substrate for the caspase-1 in cells at the site of inflammation (17). As such, if the biological effect of IL-37 is dependent on caspase-1 for its ability to suppress LPS-induced cytokines, bone marrow-derived macrophages deficient in the inflammasome components ASC and NLRP3 (9) would then exhibit no decreased response to LPS. Similar to IL-37–transfected RAW cells, production of IL-6 and IL-1β after LPS stimulation was significantly reduced in WT bone marrow-derived macrophages expressing IL-37 compared with mock-transfected controls. In contrast, IL-37 transfection into bone marrow-derived macrophages isolated from ASC- and NLRP3-deficient mice did not result in reduced LPS-induced IL-6 and IL-1β in the culture supernatants. These data indicate that activation of caspase-1 by the inflammasome is required for IL-37 to exert its cytokine-suppressing functions in macrophages.

Some RAW cells do not express a fully functional inflammasome because they lack ASC, whereas others have demonstrated caspase-1–dependent activation and release of IL-1β in stimulated RAW cells (18–20). Similarly, caspase-1 was also active in RAW cells used for this study, generating mature IL-37. Interestingly, processing of IL-37 was also shown in adenovirus-transduced IL-37–expressing 293 cells, which also do not express all components of the NLPR3 inflammasome (4, 6).

When NLRP3 activation in macrophages is induced by LPS, peptidoglycans, or bacterial nucleic acids, mature IL-1β is released upon exposure to ATP (21). In the absence of ATP, macrophages stimulated with LPS synthesize large quantities of the IL-1β precursor but only process and release less than 20% of the mature cytokine into cell culture supernatants (22, 23). Here, we show that ATP also induces the secretion of mature IL-37 from RAW–IL-37 cells and PBMCs. The release of mature IL-37 is dependent on caspase activity, because treatment with a pan-caspase inhibitor prevented nearly all externalization of mature IL-37 in transfected RAW cells. In contrast, the release of precursor IL-37 after ATP treatment is independent of caspase-1, because there was no reduction by a pan-caspase inhibitor. This is of particular interest because the recombinant IL-37 precursor is functional in suppressing LPS-induced IL-6 (Fig. 6*A*).

DCs are key effector cells of the early immune response and produce IL-1β and IL-18 (12). We reported that activation of DCs is down-regulated in IL-37tg mice after LPS challenge (8), and therefore hypothesized that IL-37 is expressed in and released from human DCs. Indeed, we detected both precursor and mature IL-37 in cell lysates of activated DCs. Secretion of IL-37 from DCs was stimulated by agonist antibodies, whereas resting DCs did not release IL-37. Similar to RAW–IL-37 cells, precursor and mature forms of IL-37 were detected in the supernatant of stimulated DCs, indicating that both caspase-1–independent release of precursor IL-37 and caspase-1–dependent secretion of mature IL-37 are physiological events in transfected and primary human cells. Immunofluorescence microscopy showed that IL-37 polarizes toward the immunological synapse after interaction with NK cells. The action of cytokines and chemokines released to the cell–cell interaction site is well established in NK cells and DCs (24). We therefore propose that membrane-bound or released IL-37 also interacts with cellular receptors at the immunological synapse in an intra- or paracrine fashion to modulate the immune response.

It is not fully understood whether intracellular IL-37 or secreted IL-37 mediates the reduction in inflammation in IL-37tg mice (8). An indication for the extracellular functionality of IL-37 was reported by Sakai et al. (13), showing that a recombinant IL-37a isoform with an N terminus at E-16 is protective in a mouse model of liver ischemia/reperfusion. To confirm that extracellular IL-37 is functional in vivo, we treated WT and IL-37tg mice with monoclonal or polyclonal neutralizing antibodies against IL-37 before LPS challenge. Both antibodies were raised against the IL-37a isoform. The polyclonal anti–IL-37 specifically stained recombinant IL-37b protein on Western blotting (Fig. S2). We first determined whether the monoclonal anti–IL-37 neutralized the recombinant IL-37 precursor by reversing IL-37–mediated suppression of LPS-induced IL-6 by M1 macrophages (Fig. 6*A*). Second, in IL-37tg mice, the mAb against IL-37, as well as a polyclonal anti–IL-37, also abrogated the protective effect of IL-37 in a model of LPS-induced endotoxemia. Because there was no effect of IL-37 antibodies on the LPS response in WT mice, these studies demonstrate that the antibodies are specific for neutralizing extracellular IL-37 in inflammation. Although these findings indicate that IL-37tg mice release biologically active IL-37, we cannot discriminate whether extracellular IL-37 released in vivo is the precursor or a post-caspase-1–cleaved mature cytokine. We have observed that the precursor form of IL-37, as well as the mature cytokine, is active in suppressing LPS-induced IL-1β, IL-6, and TNF-α from M1 macrophages in vitro (25).

A candidate receptor for extracellular IL-37 is the IL-18 receptor α (IL-18Rα) (4, 6, 26). However, despite binding to the IL-18Rα, we, as well as others, could not demonstrate that IL-37 functions as a receptor antagonist for IL-18 (4, 27). Instead, it is likely that once IL-37 binds to IL-18Rα, an anti-inflammatory coreceptor, such as single Ig IL-1–related receptor/IL-1R8 (26), is recruited (Fig. 7). Although anti–IL-37 enhanced the LPS-induced systemic inflammation in IL-37tg mice, the neutralizing antibody did not alter the IL-6 response in LPS-stimulated IL-37–expressing RAW cells. This observation is consistent with a predominant intracellular function of IL-37 in transfected RAW cells through binding to Smad3 and translocation to the nucleus, as we previously described (8) (Fig. 7).

In summary, we show here that caspase-1 processing is required for maturation of the intracellular IL-37 precursor and for the translocation of the cytokine to the nucleus. Caspase-1 is also required for the secretion of mature IL-37. In contrast, the release of the IL-37 precursor from LPS-stimulated macrophages is independent of caspase-1 activation. IL-37 is not active in cells

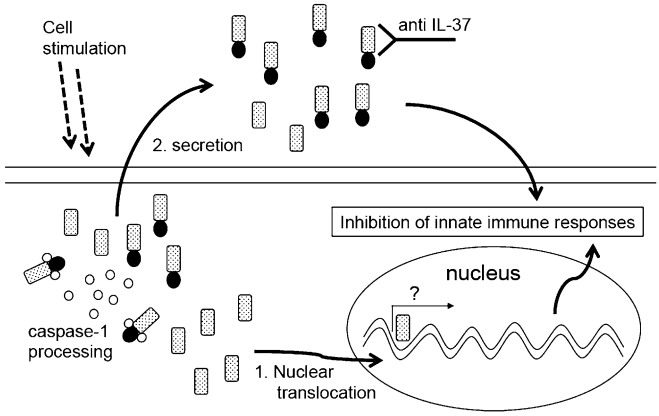

Fig. 7. IL-37 mechanism of action as a dual-function cytokine.

from ASC- and NLRP3-deficient mice, indicating that the function of IL-37 is NLRP3 inflammasome-dependent. In vivo, we observed that IL-37 is biologically active when released into the extracellular space, because neutralizing antibodies reversed the anti-inflammatory properties in IL-37tg mice. Thus, IL-37 emerges as a dual-function cytokine with intracellular and extracellular mechanisms of action.

Materials and Methods

Reagents. LPS (*Escherichia coli* 055:B59) was purchased from Sigma. The pan-caspase inhibitor Z-VAD-(OMe)-FMK was purchased from R&D Systems. Cytokine determinations were made with an ELISA purchased from R&D Systems.

Mutation of Caspase-1 Cleavage Site in IL-37. Using the most abundant isoform b of IL-37, we generated the IL-37D20A mutant by site-directed mutagenesis as detailed in *SI Materials and Methods*.

Generation of Stable RAW Cells. RAW cells were transfected with IL-37D20A and IL-37D20A–YFP or CFP–IL-37D20A fusion constructs (*SI Materials and Methods*) and subcloned by limiting dilution. Mock-transfected RAW cells were generated as described (28).

LPS Stimulation of Transfected RAW Cells, PBMCs, and M1 Macrophages. Stable transfectants of RAW cells expressing IL-37 and mock-transfected cells were stimulated with LPS as previously described (10, 11). Details on cell fractionation and nuclear extraction are described in *SI Materials and Methods*. M1 macrophages were differentiated from human PBMCs as described in *SI Materials and Methods*.

Generation and Immunofluorescence Analysis of Activated DCs and NK Cells. NK cells were isolated from PBMCs from healthy donors as described elsewhere (12). DCs were generated from monocytes isolated from PBMCs as described previously and in detail in *SI Materials and Methods*.

Transfection of IL-37 into ASC- and NLRP3-Deficient Macrophages. Immortalized WT, ASC-deficient, and NLRP3-deficient bone marrow macrophages were the generous gift of Eicke Latz (Institute of Innate Immunity, Bonn, Germany) (9, 29). IL-37b in pIRES vector (BD Clontech) (8) and pIRES without insert were used for transfection. Cells were transfected using an Amaxa Nucleofector Kit V and Program X-001 (Lonza AG). After transfection, cells were transferred to 24-well plates (25,000 cells per well) and allowed an overnight rest period in DMEM supplemented with 10% (vol/vol) FCS and 1% penicillin/streptomycin. On the next day, the medium was replaced with reduced serum (1%) DMEM and the cells were stimulated with either vehicle or LPS (10 ng/mL; *E. coli* 055:B5). At the end of each experiment, supernatants were taken and cells were lysed in lysis buffer and stored at −80 °C.

Animals. Animal protocols were approved by the review board of the Federal Government of Bavaria, Gemany (Az. 55.2.1.54-2532-77-11). Control male C57/BL6 mice were purchased from Janvier (France). Transgenic C57/BL6 mice expressing the human IL-37 precursor (IL-37tg) have been described previously (8).

Serum Levels of IL-6 Following LPS Injected in Mice. Eight-wk-old male IL-37tg mice and male WT C57/Bl6 mice were injected i.p. with a mAb against IL-37 (R&D Systems), affinity-purified goat anti–IL-37 (R&D Systems), or control goat IgG (R&D Systems). After 2 h, mice received 100 μg of LPS i.p., and after 4 h and 18 h, blood was collected and serum IL-6 was determined by ELISA (BD Biosciences).

Western Blotting. Cell lysates were subjected to SDS/PAGE under reducing conditions. For detection of IL-37 protein, mAb against IL-37 was used. Details of Western blotting are described in *SI Materials and Methods*.

Confocal Microscopy. RAW cells transfected with pEYFP-N1–IL-37 fusion construct were analyzed using a confocal laser scanning microscope (LSM510Meta; Carl Zeiss) as described (5).

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Supporting Information

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SI Materials and Methods

Mutation of Caspase-1 Cleavage Site in IL-37. Mutant IL-37D20A was generated by site-directed mutagenesis. The template of the first PCR assay was WT IL-37 isoform b in pTarget (1). The 5′ region was amplified using a sense vector primer, 5′-CGTCG-CCGTCCAGCTCGACCAG-3′, that binds to the upstream region of the ORF and a reverse primer, 5′-ATGGGCTCTGAG-GACTGGGAAAAAGCTGAA-3′, that corresponds to the Asp20 (GAT)-containing region to switch Asp20 to Ala20 (GCT). The 3′ region was amplified with a sense primer, 5′-CATGGTCCT-GCTGGAGTTCGTG-3′, that corresponds to the Asp region to mutate Asp20 to Ala20 and a reverse vector primer, 5′-GTCT-TCTAAGCAGCACTGGGGTTCAGCTTT-3′, that binds to a downstream region of the ORF. The PCR products were purified using a Gel Purification Kit (COSMO). For the second PCR assay, purified PCR products were mixed at a 1:1 molar ratio and a sense primer, 5′-TTTACTCGAGCCACCATGTCCTTTGT-GGGGGAG-3′, that corresponds to 5′ of the ORF with the initiation codon and a reverse primer corresponding to the 3′ of the ORF with or without the termination codon were used as specific restriction sites for cloning into the expression plasmids. IL-37D20A cDNA was cloned into p-Target, p-enhanced CFP (pECFP)-C1 and p-enhanced YFP (pEYFP)-N1 expression plasmids (BD Clontech). All plasmids were isolated by means of a "low LPS" MaxiPrep kit (Qiagen).

Cell Culture. All cells were grown in RPMI 1640 supplemented with 5% heat-inactivated FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Life Technologies) at 37 °C in a 5% CO_2 incubator.

Generation of Stable RAW 264.7 Transfectants. RAW 264.7 (RAW) cells were transfected with IL-37D20A in pTarget (Promega) and IL-37D20A–YFP or CFP–IL-37D20A fusion constructs in pEYFP-N1 or pECFP-C1 expression plasmid using FuGENE HD transfection reagent (Roche Applied Science). Transfected cells were selected in the culture medium supplemented with 200 μg/mL Geneticin G418 (Roth) and subcloned by limiting dilution. Mock-transfected RAW cells were generated as described (1).

LPS Stimulation of Transfected RAW Cells, Peripheral Blood Mononuclear Cells, and M1 Macrophages. Stable transfectants of RAW cells expressing IL-37 (RAW–IL-37) and mock-transfected cells were seeded in a six-well plate at 1×10^6 cells per well in growth medium at a rate of 2 mL per well and preincubated with goat anti–IL-37 IgG or control IgG (10 μg/mL; both from R&D Systems). After 4 h, cells were stimulated with LPS (100 ng/mL) for 18 h. Cell culture supernatants were analyzed for IL-6 by ELISA (BD Biosciences). For peripheral blood mononuclear cell (PBMC) and M1 macrophage study, 0.5×10^6 PBMCs were seeded per well in a 96-well flat bottom plate. After 1 h, nonadherent cells were removed by gentle rinsing and adherent cells were differentiated with 50 ng/mL human GM-CSF for 6 d as previously described (2). After differentiation, the medium was removed and the cells were pretreated with recombinant IL-37 (1 ng/mL) with or without the addition of a mouse mAb against IL-37 (50 ng/mL; R&D Systems) for 2 h before 10 ng/mL LPS was added.

Cell Fractionation. A total of 1×10^6 RAW cells expressing IL-37 or IL-37D20A were cultured with or without LPS (100 ng/mL) overnight. Cells were washed twice with ice-cold PBS and homogenized with 250 μL of 10 mM Hepes (pH 7.9), 10 mM KCl, 10% glycerol, 0.34 M sucrose, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. Nuclei were washed three times with the homogenization buffer and then lysed with 10 μL of 60 mM Tris·Cl (pH 6.8), 2% SDS, 10% glycerol containing 1 μL of 1 M $MgCl_2$, and 1 μL of Benzonase (Sigma–Aldrich). Nuclear extracts were boiled in Laemmli buffer under reducing conditions and applied to SDS gel electrophoresis and Western blotting.

Stimulation of IL-37 Release by ATP. RAW cells expressing IL-37 in pTarget were seeded in a six-well plate at 2×10^6 cells per well in 2 mL of growth medium per well. Cells were pretreated with pan-caspase inhibitor (10 μg/mL) for 1 h as indicated and stimulated with LPS (100 ng/mL) for 4 h. Cells were then washed twice with PBS and incubated with 500 μL of freshly prepared 5 mM ATP in PBS. Cells were subsequently harvested by centrifugation, and supernatants were collected, centrifuged, and subjected to a membrane-based concentration step (10 kDa of Amicon Ultracel-10 Membrane; Merck Millipore). Cells were washed twice with cold PBS and then lysed in 500 μL of 50 mM Hepes (pH 7.8), 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor (Thermo Scientific). Lysates were incubated on ice for 30 min and then clarified by centrifugation at $13{,}000 \times g$ for 30 min at 4 °C.

Generation and Immunofluorescence Analysis of Activated Dendritic and Natural Killer Cells. Dendritic cells (DCs) were generated from monocytes isolated from PBMCs by adherence and incubated with GM-CSF and IL-4 for 7 d. DCs were incubated in RPMI medium supplemented with 1% Nutridoma-HU (Sigma–Aldrich), in the presence or absence of anti-CD54 mAb (intercellular adhesion molecule 1, clone SM89, IgM; Serotec), anti-CD94 mAb (HP-3B1, IgG2a; Serotec), or anti-HLA I class-I (W6/32, IgG2a; American Type Culture Collection) for 6 h. At the end of the stimulation period, culture supernatants were collected and concentrated with trichloroacetic acid and cells were lysed in Triton X-100 for Western blot analysis. In coculture experiments, DCs were incubated with autologous natural killer (NK) cells at an NK cell/DC ratio of 5:1 for 6 h before processing for immunofluorescence. After 6 h of coculture, NK cell/DC conjugates were fixed and permeabilized with 3% paraformaldehyde and 0.5% Triton X-100. After washings and saturation with 2% BSA, cells were labeled with a mouse anti–IL-37 mAb (3) and antitubulin antibody, followed by incubation with the appropriate secondary reagents labeled with cyanine 3 (Cy3; Jackson ImmunoResearch) and FITC, respectively. Cells were examined under an Olympus AX70 microscope using a 100/1.30 oil objective lens. Images were acquired with a Hamamatsu digital camera.

Animals and Animal Housing. Animals were housed at a controlled temperature with light/dark cycles with free access to food and water and were acclimatized for 2 wk before being studied. The monoclonal anti–IL-37 injected into IL-37 transgenic mice was shown to block the binding of a recombinant IL-18 receptor α/Fc construct to immobilized IL-37 and also detects IL-37 on Western blots (Data Sheet MAB1975; R&D Systems).

Recombinant Human IL-37. Full-length human IL-37b isoform was inserted in the expression plasmid pCACTUS with a chicken β-actin promoter and N-terminal 6-histidines. After expression in *Escherichia coli*, the recombinant molecule was purified on Talon, followed by FPLC size exclusion. The peak isolated from the FPLC was applied to a C6 HPLC column, and the IL-37 peak was eluted

in acetonitrile, isolated, and lyophilized. The lyophilized IL-37 was reconstituted in PBS.

Western Blotting. Cell lysates were separated, subjected to SDS/PAGE under reducing conditions on an SDS polyacrylamide gel (Any kDa Resolving Gel; Bio-Rad) under reducing conditions, and transferred onto a PVDF membrane (Immun-Blot PVDF Membrane; Bio-Rad). The blots were blocked in 5% nonfat dry milk in PBS and 0.1% Tween 20 for 1 h at room temperature. For detection of IL-37 protein, a mouse mAb against IL-37 was incubated overnight at 4 °C in blocking buffer (3). Alternatively, we used a polyclonal goat anti–IL-37 IgG (1 μg/mL; R&D Systems). After washing, the membrane was incubated with a secondary horseradish-conjugated anti-mouse or anti-goat antibody (Sigma–Aldrich) at room temperature for 1 h diluted in blocking buffer. The membranes were developed with an enhanced chemiluminescence reagent (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare). For detection of IL-37 in cytoplasmic extracts and supernatants of DCs cocultured with NK cells, aliquots of cell lysates, trichloroacetic acid-concentrated supernatants, and cytoplasmic protein fractions were boiled in reducing Laemmli sample buffer; resolved on 12% SDS/PAGE; and electrotransferred onto a PVDF membrane. Filters were hybridized with a mouse anti-human IL-37 mAb (1) or antitubulin antibody (Sigma–Aldrich), followed by goat anti-mouse HRP-conjugated secondary reagent (Dako), and were developed using ECL Plus (Amersham Biosciences), according to the manufacturer's instructions.

Statistical Analysis. Results are expressed as mean ± SEM. Differences between control and treated groups were compared by the Mann–Whitney *U* test. Statistical analysis was performed with Prism 5, version 5.0f for Macintosh (GraphPad).

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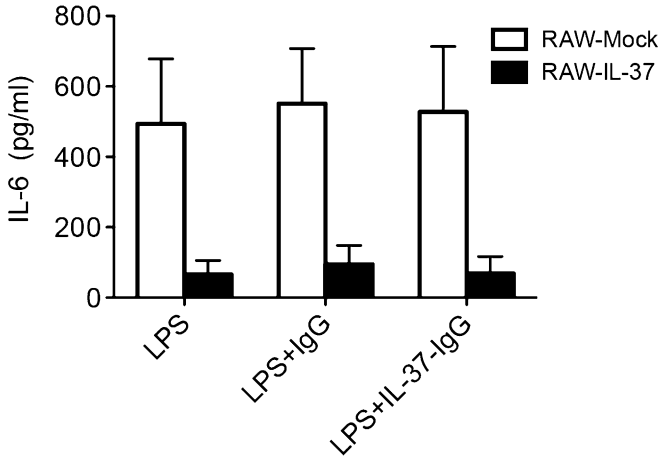

Fig. S1. Anti–IL-37 does not modulate LPS-induced IL-6 secretion by IL-37–transfected RAW cells. RAW–IL-37 cells and mock transfectants were pretreated with control IgG or an anti–IL-37 antibody (10 μg/mL) and then stimulated with LPS (100 ng/mL) for 18 h. Mean ± SEM supernatant IL-6 levels of three independent experiments are shown.

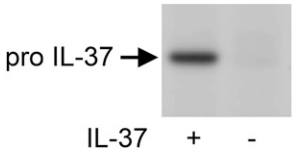

Fig. S2. Staining of recombinant IL-37 on a Western blot by polyclonal anti–IL-37. The cell lysate of RAW transfectants expressing either IL-37 or control plasmid was separated on SDS/PAGE. Western blotting was performed with a goat polyclonal anti–IL-37 IgG (R&D Systems).